

Original Article

Early establishment of *Bifidobacterium*-dominant microbiome in planned cesarean-born infants via *Bifidobacterium breve* strain Yakult supplementation: A randomized, double-blind, placebo-controlled, parallel-group pilot study[☆]

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ABSTRACT

Objectives: To investigate the effects of *Bifidobacterium breve* strain Yakult (BbrY) administration immediately after birth on the microbiome of cesarean-born full-term infants with an immature microbiota at birth.

Methods: This single-center, placebo-controlled, randomized, double-blind, parallel-group intervention study included healthy, full-term Japanese infants born via planned cesarean section. Infants were administered either BbrY or placebo for the first month of life. The primary endpoint was the early establishment of a *Bifidobacterium*-dominant microbiome.

Results: This study included 26 infants (14 in the BbrY group and 12 in the placebo group). Compared with the placebo group, the BbrY group showed significantly increased detection rates and counts of BbrY from day three to six months post-birth, with the level and proportion of *Bifidobacterium* and *Bifidobacteriaceae* remaining elevated until one month post-birth. Total bacterial counts and total organic acid concentrations, including acetic and lactic acids, were significantly higher in the BbrY group until the sixth day of life, concomitant with a decrease in fecal pH. At nine months, the BbrY group exhibited a significantly higher body weight than the placebo group, and the Kaup index remained within the normal range at six and nine months.

Conclusions: BbrY supplementation immediately after birth facilitated the early establishment of a *Bifidobacterium*-dominant microbiome and contributed to intestinal acidification in cesarean-born infants, indicating BbrY administration may support normal growth patterns during infancy.

1. Introduction

Cesarean section accounted for 21.1% of births globally in 2018, with projections suggesting an increase to 28.5% by 2030 [1]. The substantial rise in cesarean section rates over recent decades has been attributed to factors such as increasing maternal age, preferences of

healthcare providers, and choice of expectant mothers. Although the rate of cesarean section in Japan has not been reported internationally, a survey conducted in 2013 estimated it at 18.5% [2]. Considering the global trend of rising cesarean rates and associated contributing factors, a similar increase in cesarean births is anticipated in Japan. Infants born via cesarean section experience delayed establishment of the intestinal microbiota owing to the absence of exposure to maternal vaginal and

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Abbreviations

BbrY	<i>Bifidobacterium breve</i> strain Yakult
16S rRNA	16S ribosomal RNA
GL	Galactosyl lactose
PBS	Phosphate-buffered saline

intestinal microbial transfer during delivery [3–5]. This delay particularly affects colonization by bifidobacteria, which are critical for the early development of healthy gut microbiota and have been linked to long-term health consequences, including an increased risk of immune-mediated diseases [6–9]. Therefore, addressing the delayed development of the intestinal microbiota in infants born by cesarean section is a key focus in neonatal health research.

Bifidobacterium dominates the intestinal microbiota of infants born vaginally, and *Bifidobacterium breve* is among the most prevalent species [10]. Probiotic supplementation with the *B. breve* strain Yakult (BbrY) has demonstrated efficacy in establishing an early bifidobacteria-dominant microbiota, improving the intestinal environment, and promoting growth in preterm and low-birth-weight infants [11,12]. Furthermore, its safety has been well established, with only four cases of adverse events reported among approximately 20,000 cases of clinical use of BbrY [13,14]. However, its potential to support early colonization of the gut microbiota in healthy, full-term cesarean-born infants remains unexplored.

This randomized, double-blind, placebo-controlled study aimed to evaluate the effects of BbrY administration on the early establishment of bifidobacteria-dominant gut microbiota in healthy full-term Japanese infants delivered via planned cesarean section. These findings have significant implications for mitigating microbiota-related health risks in cesarean-born infants and could inform guidelines for probiotic administration in neonatal healthcare.

2. Materials and methods

2.1. Subjects and study design

The study included healthy male and female infants born by planned cesarean section at Gonohashi Obstetrics and Gynecology Hospital (Koto-ku, Tokyo, Japan) between July 2019 and November 2022. Written informed consent was obtained from the parents or legal guardians after they were provided with a full explanation of the study's purpose and procedures. The study was approved by the Nihonbashi Cardiology Clinic Study Review Committee (approval number: NJI-018-03-02) and the Juntendo University Hospital Ethics Committee (approval number: 180-113) and was conducted under physician supervision in accordance with the Declaration of Helsinki. This study was registered in the University Hospital Medical Information Network Research Center public database (UMIN Study ID: UMIN000036526).

This randomized, double-blind, placebo-controlled, parallel-group study evaluated two test foods: BbrY supplement and placebo. BbrY supplement was provided by Yakult Honsha Co. Ltd. (Minato-ku, Tokyo, Japan). It was packaged as a freeze-dried powder mixed with corn starch in 1 g sachets containing no less than 10^9 colony-forming units of BbrY. The placebo consisted of corn starch alone and was provided in sachets identical in appearance to those containing BbrY supplement. The primary endpoint of this study was the early establishment of a *Bifidobacterium*-dominant microbiome in cesarean-born infants. The secondary endpoints included changes in the intestinal microbiota composition, intestinal environment (e.g., pH and organic acids), and assessments of growth and physical health. The participants received a daily dose of the intervention from immediately after birth to one month of age. A sterile plastic container containing sterile distilled water (7.5

mL) was used to mix either one sachet of BbrY supplement or placebo (1 g). After standing for at least 1 min, 1 mL of the supernatant (equivalent to $8.1 \log_{10}$ colony-forming units/day) was administered orally using a sterile plastic dropper.

2.2. Sample collection and analysis

Stool samples (approximately 0.2–0.5 g) were collected at birth and on days 3 and 6, as well as at 1, 3, and 6 months of age. Samples were collected either in empty fecal collection tubes (Sarstedt AG & Co., Nümbrecht, Germany) for bacterial count, DNA extraction, pH measurement, and organic acid concentration analysis, or in tubes containing 2 mL of RNAlater (Thermo Fisher Scientific KK, Waltham, MA, USA) for RNA extraction.

Fecal samples were collected at the hospital (on days 1, 3, and 6) and frozen immediately at -30°C . Samples collected at participants' homes at 1 month and later were stored at 4°C immediately after collection and transported to the laboratory under controlled conditions within 12 h. Upon arrival, these samples were then frozen at -30°C for short-term storage and subsequently transferred to -80°C for long-term preservation.

2.3. Physical examinations and monitoring

Medical examinations were performed by a physician on day 6 and 1, 3, 6, 9, and 12 months of age. Measurements included the head circumference, weight, and height. The Kaup index (weight [g]/height [cm] $[2] \times 10$) was calculated based on these measurements, following established guidelines [15]. Throughout the 12-month study period, parents or guardians maintained daily records of the health status of infants, medication use, and diet type. Blinding and management of the study participants were conducted independently by K.S.O. Inc. (Minato-ku, Tokyo, Japan) to ensure unbiased administration and evaluation.

2.4. Primary treatment of feces and measurement of pH

Frozen stool samples were transported to the Yakult Central Institute (Kunitachi-shi, Tokyo, Japan) under controlled conditions and stored at -80°C until further processing. Stool samples collected in empty fecal collection tubes were thawed, and pH was measured using a D-52 pH meter (Horiba, Ltd., Kyoto, Japan). Stool weight was recorded prior to additional processing. A 10-fold dilution of stool was prepared using phosphate-buffered saline (PBS). From this diluted solution, 200 μL was transferred to new 2 mL tubes for DNA extraction and stored at -80°C ; 450 μL was allocated for organic acid measurement and similarly stored at -80°C . To quantify the total bacterial count, 100 μL of the diluted stool sample was combined with 300 μL of 4% paraformaldehyde, fixed at 4°C for 16 h, and stored at -80°C . For RNA extraction, stool samples were diluted 50-fold with RNAlater solution. A 200 μL aliquot of the diluted sample was transferred to a new tube and centrifuged at $13,000 \times g$ for 5 min at 4°C to remove the supernatant, and the resulting pellet was stored at -80°C for later use.

2.5. DNA extraction, 16S rRNA gene sequencing, and microbiota analysis

DNA was extracted from a 10-fold dilution of stool samples using the bead phenol method, and 16S rRNA amplicon sequencing was performed following the protocol described in a previous study [16]. The variable region V1–V2 of the bacterial 16S rRNA gene was amplified from the extracted DNA using primers 27Fmod2-MiSeqV2 (5'-AATGATACGGGCGACCGAGATCTACACTCTTCCCTACACGACGCTC-TTCCGATCT-AGRGTTYGATYMTGGCTCAG-3') and 338R

MiSeq (5'-CAAGCAGAAGACGGCATACGAGAT-NNNNNNNNNN-GTGACTGGAGTTCAGACGTGCTCTTCCGATCT-GCTGCCWCCCGTAGGGWGT-3'). The resulting amplicons were

sequenced using the MiSeq platform (Illumina), and the obtained sequences were analyzed using QIIME2 software (ver2022.2; <https://qiime2.org/>). Low-quality reads were eliminated, and 16S rRNA reads were denoised, concentrated, and checked for potential chimeric sequences using the DADA2 algorithm (<https://benjjneb.github.io/dada2/>). Taxonomic classification of the amplicon sequence variants (ASVs) was performed using the QIIME2 ASVs classifier plugin, referring to the SILVA database (release 138.1; <https://www.arb-silva.de/documentation/release-1381/>). A minimum sampling depth of 20,541 reads was used to determine the sequence concentration. Diversity analyses, including α -diversity indices, such as Observed ASVs, Shannon index, Faith's PD, and Pielou's evenness, were conducted using the QIIME2 diversity plugin. To quantify the bacterial abundance at the family level, compositional data for the top ten families were multiplied by the total bacterial count. The newborn stool samples collected immediately after birth did not yield a sufficient amount of DNA to amplify the V1–V2 region of 16S rRNA; therefore, these samples were excluded from the analysis.

2.6. Quantification of BbrY

BbrY counts were quantified using quantitative PCR (qPCR) with BbrY-specific primers (pBbrY-F, 5'-ATGGCAAACCGGGGCTGAA-3'; pBbrY-R, 5'-GCGGATGAGAGGTGGG-3') [17]. DNA extracted from stool samples was used as the template for amplification. The presence of BbrY was confirmed when amplification occurred with specific primers, and the amplified ASVs from MiSeq sequencing were 100% identical to the 16S rRNA gene sequence of BbrY (GenBank accession number: PV426328). Stool samples with BbrY levels below the detection limit of 106.0 cells/g feces were substituted with a value of 103.0 cells/g feces, which represented half of the detection limit.

2.7. RNA extraction and quantification of major bacterial groups

Total RNA was extracted from 4 mg of feces using the acid phenol method, according to a previous report [18], and dissolved in 200 μ L nuclease-free water. The extracted total RNA was used as a template for reverse-transcription quantitative PCR (RT-qPCR) to quantify the counts of the following groups, genera, and species: in stool samples immediately after birth (meconium), the counts of the *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Bacteroides fragilis* group, *Bifidobacterium*, *Atopobium* cluster, *Prevotella*, *Clostridium perfringens*, *Clostridioides difficile*, *Lactobacillus*, *Levilactobacillus brevis*, *Lactocaseibacillus*, *Limosilactobacillus fermentum*, *Fructilactobacillus fructivorans*, *Lactiplantibacillus*, *Limosilactobacillus*, *Liquorilactobacillus*, *Ligilactobacillus*, *Latilactobacillus*, *Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Pseudomonas* were measured. The counts of *Bifidobacterium*, *C. perfringens*, *C. difficile*, *Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Pseudomonas* were measured in stool samples collected from 3 days to 6 months of age. Values below the detection limit were substituted with half of the detection limit values. The detection limits and their corresponding half-values ($\log_{10}$ cells/g feces) were as follows: *C. coccoides* group: 5.0, *C. leptum* subgroup: 5.0, *B. fragilis* group: 5.0, *Bifidobacterium*: 4.9, *Atopobium* cluster: 4.9, *Prevotella*: 5.1, *C. perfringens*: 2.3, *C. difficile*: 2.3, total lactobacilli: 2.3, *Lactobacillus*: 2.3, *L. brevis*: 2.3, *Lactocaseibacillus*: 3.0, *L. fermentum*: 2.9, *F. fructivorans*: 2.3, *Lactiplantibacillus*: 2.3, *Limosilactobacillus*: 2.3, *Liquorilactobacillus* and *Ligilactobacillus*: 2.3, *Latilactobacillus*: 3.0, *Enterobacteriaceae*: 4.0, *Enterococcus*: 3.0, *Streptococcus*: 3.0, *Staphylococcus*: 3.0, and *Pseudomonas*: 3.0).

2.8. Measurement of total bacterial counts

Total bacterial counts in fecal samples were determined using a CytoFLEX S flow cytometer (Beckman Coulter, Miami, FL, USA) with Precision Count Beads (BioLegend, San Diego, CA, USA; 1.03×10^6

particles/mL) as internal standards. Fecal samples fixed with para-formaldehyde were diluted 2-fold in phosphate-buffered saline (PBS) and filtered through a #200 nylon mesh (ASONE, Osaka, Japan). Serial five-fold dilutions of the filtrate (final dilutions of 80-, 400-, 2000-, and 10,000-fold) were prepared in PBS. In 96-well plates, 10 μ L of each fecal dilution was mixed with 10 μ L Precision Count Beads (BioLegend), 30 μ L PBS, and 150 μ L PBS containing SytoBC (diluted 3000- or 6000-fold; Thermo Fisher Scientific, Eugene, OR, USA). The mixture was incubated on ice for 15–60 min before analysis using a CytoFLEX S flow cytometer (Beckman Coulter) to generate fluorescence-activated cell sorting data. Data were analyzed using CytoExpert v2.4 software (Beckman Coulter). Total bacterial counts (cells/g feces) were calculated based on the ratio of SytoBC-positive bacterial counts to the number of internal standard beads using the following formula:

$$\text{Total bacteria (cells/g feces)} = \text{Sample dilution} \times \left(\frac{\text{Beads volume}}{\text{Sample volume}} \right) \times \left(\frac{\text{SytoBC-positive count}}{\text{Beads count}} \right) \times 1.03 \times 10^6$$

For values below the detection limit ($10^{7.3}$ cells/g feces), half of the detection limit value ($10^{3.65}$ cells/g feces) was substituted.

2.9. Measurement of the concentration of fecal organic acids

The concentrations of organic acids in fecal samples were measured as previously described [19]. Briefly, a 10-fold diluted fecal sample was deproteinized with perchloric acid. The supernatant obtained after centrifugation ($12,000 \times g$ for 10 min at 4 °C) was filtered through a 0.45 μ m Centricut Ultramini filter (Kurabo Ltd., Osaka, Japan). The filtered supernatant was used to estimate the concentrations of succinic, lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids using a high-performance liquid chromatography system (Waters, Milford, MA, USA). The total organic acid concentration was determined as the sum of the concentrations of individual organic acids. For values below the detection limit, half of the detection limit value was substituted (succinic acid: 0.1 μ mol/g feces; lactic acid: 0.1 μ mol/g feces; formic acid: 0.1 μ mol/g feces; acetic acid: 0.1 μ mol/g feces; propionic acid: 0.1 μ mol/g feces; isobutyric acid: 0.2 μ mol/g feces; butyric acid: 0.2 μ mol/g feces; isovaleric acid: 0.1 μ mol/g feces; valeric acid: 0.1 μ mol/g feces).

2.10. Statistical analysis

Statistical analyses were conducted to compare variables between the two study groups. Normality was assessed using the Shapiro–Wilk test. Student's t-test was applied to normally distributed variables, whereas the Mann–Whitney U test was used for non-normally distributed variables. Spearman's rank correlation analyses were conducted to examine associations between BbrY counts and markers of intestinal acidification, including fecal pH, total organic acids, acetic acid, and lactic acid, at postnatal days 3 and 6, and month 1. For variables such as microbial count, α -diversity, organic acid concentration, and pH, the Mann–Whitney U test was applied. Fisher's exact probability test was used for categorical variables, including sex, frequency of medication use, frequency of illness, and type of diet. Mixed-effects models [20] were used to evaluate changes in head circumference, height, weight, and the Kaup index over time. All statistical analyses were performed using R software (version 4.2.3; <https://cran.r-project.org/>), and the tidyverse, lme4, lmerTest, broom.mixed, and emmeans packages were used. A p-value of less than 0.05 was considered statistically significant, while a p-value between 0.05 and 0.1 was regarded as indicative of a significant trend.

3. Results

3.1. Characteristics of study participants

Twenty-seven participants (14 in the BbrY group and 13 in the placebo group) were initially included in this study. However, one participant was excluded after a vaginal delivery occurred following consent, resulting in 26 subjects (14 in the BbrY group and 12 in the placebo group) who completed the study (Fig. 1). The adherence rate to the BbrY supplement and placebo was $98.8 \pm 0.7\%$ up to one month of age, with no intake-related adverse events reported through 12 months of age. None of the participants met the exclusion criteria; therefore, all 26 participants were included in the efficacy analysis. No significant differences were observed in gestational age, birth height, or head circumference between the BbrY and placebo groups. Birth weight tended to be higher in the placebo group ($p = 0.06$) (Table 1). A greater proportion of participants in the placebo group tended to consume formula milk at 3 months than those in the BbrY group ($p = 0.09$). However, the food intake frequency did not show significant differences between the groups throughout the study period (Supplementary Table 1). Feeding patterns were assessed on day 6 and months 1, 3, 6, 9, and 12 (Supplementary Fig. 1). Exclusive breastfeeding rates were 14.3% vs. 25.0% on day 6; 7.1% vs. 33.3% at 1 month; and 14.3% vs. 50.0% at 3 months, in the BbrY and placebo groups, respectively. Formula feeding at 3 months was reported in 85.7% of participants in the BbrY group and 50.0% in the placebo group. Complementary foods were introduced at a median age of 6 months in both groups. None of these differences was significant.

Analysis of the bacterial counts, detection rates of fecal microbiota, and organic acid concentrations in meconium samples revealed no significant differences between the two groups in bacterial counts or detection rates for any intestinal bacterial group analyzed by RT-qPCR (Supplementary Table 2). Nevertheless, organic acid concentrations in the meconium showed a trend toward higher acetic acid levels in the placebo group than that in the BbrY group ($p = 0.06$). No significant differences were observed in the concentrations of the other organic acids between the two groups (Supplementary Table 3).

3.2. Bifidobacterium-dominant microbiome in early infancy by BbrY consumption

Fig. 2A shows the counts and detection rates of BbrY. In the BbrY group, BbrY was detected in 100% of the participants from day three to the first month of life, with counts exceeding 1×10^{10} cells/g feces. After three months, both the detection rate and BbrY count decreased; however, at six months of age, BbrY was still detected in 79% of the participants. In contrast, in the placebo group, BbrY was not detected between day three and three months of life, and at six months, both the detection rate and count were significantly lower than those in the BbrY group.

Fig. 2B shows the counts and detection rates of *Bifidobacterium* spp. From day three to six months, the BbrY group consistently showed nearly 100% detection of *Bifidobacterium*, whereas in the placebo group, *Bifidobacterium* was not detected in one-third of the participants, even in the first month. The bifidobacterial count in the BbrY group was significantly higher or tended to be higher than that in the placebo group from day three to the first month of life.

Fig. 2C depicts the change in the average relative abundance of the *Bifidobacteriaceae* family based on 16S rRNA amplicon sequencing analysis. In the BbrY group, *Bifidobacteriaceae* constituted 60–70% of the microbiome from day three to six months of age. In contrast, the placebo group showed approximately 50% relative abundance even at one month, with *Bifidobacteriaceae* levels significantly lower than those in the BbrY group from day three to the first month.

3.3. Effect of BbrY consumption on microbiome and environment development in early infancy

Supplementary Fig. 2 illustrates the changes in total bacterial counts and relative abundance of major bacterial families, excluding those showing no significant differences or trends between the groups. The BbrY group exhibited significantly higher total bacterial counts or trends toward higher counts at 3–6 days of age than the placebo group. By the first month, the *Bifidobacteriaceae* family counts in the BbrY group were significantly higher than those in the placebo group. Additionally, the BbrY group showed a trend toward higher counts of *Staphylococcaceae* at

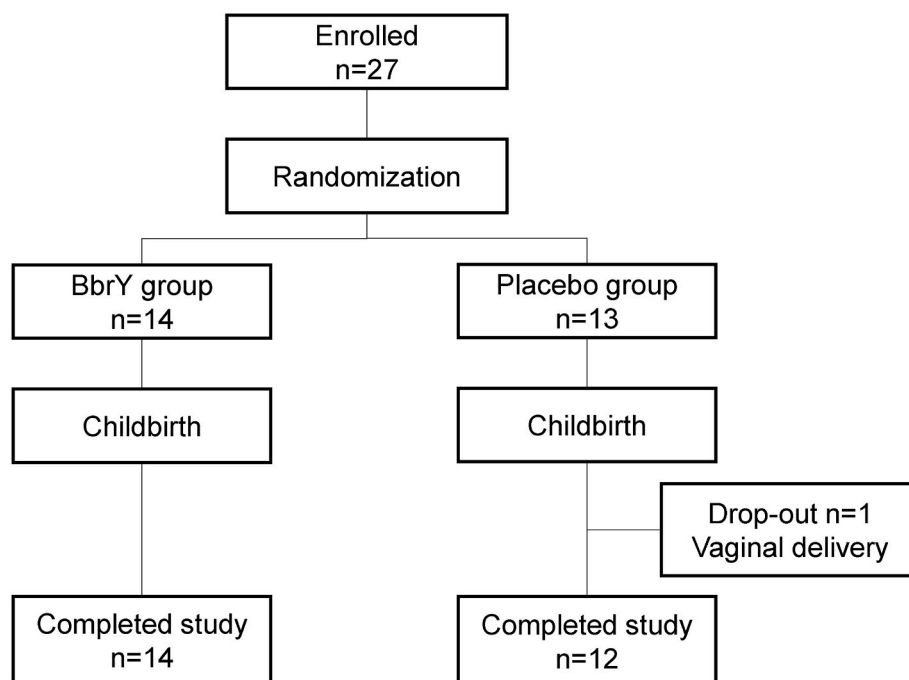


Fig. 1. CONSORT flow diagram of the selection of participants for the study.

Table 1
Characteristics of study subjects.

Factor			Placebo	BbrY	P
Gender	(%)	male	7 (58.3)	6 (42.9)	0.70
		female	5 (41.7)	8 (57.1)	
Gestational age	day		267.50 (4.42)	267.79 (4.12)	0.87
Birth head circumference	cm		34.08 (1.38)	33.72 (1.18)	0.48
Birth weight	g		3138.67 (422.70)	2832.00 (357.46)	0.06
Birth height	cm		48.24 (1.79)	47.74 (2.04)	0.52

The values represent means (standard deviation); p-values were calculated using the Student's t-test (two-tailed) (#p < 0.1).

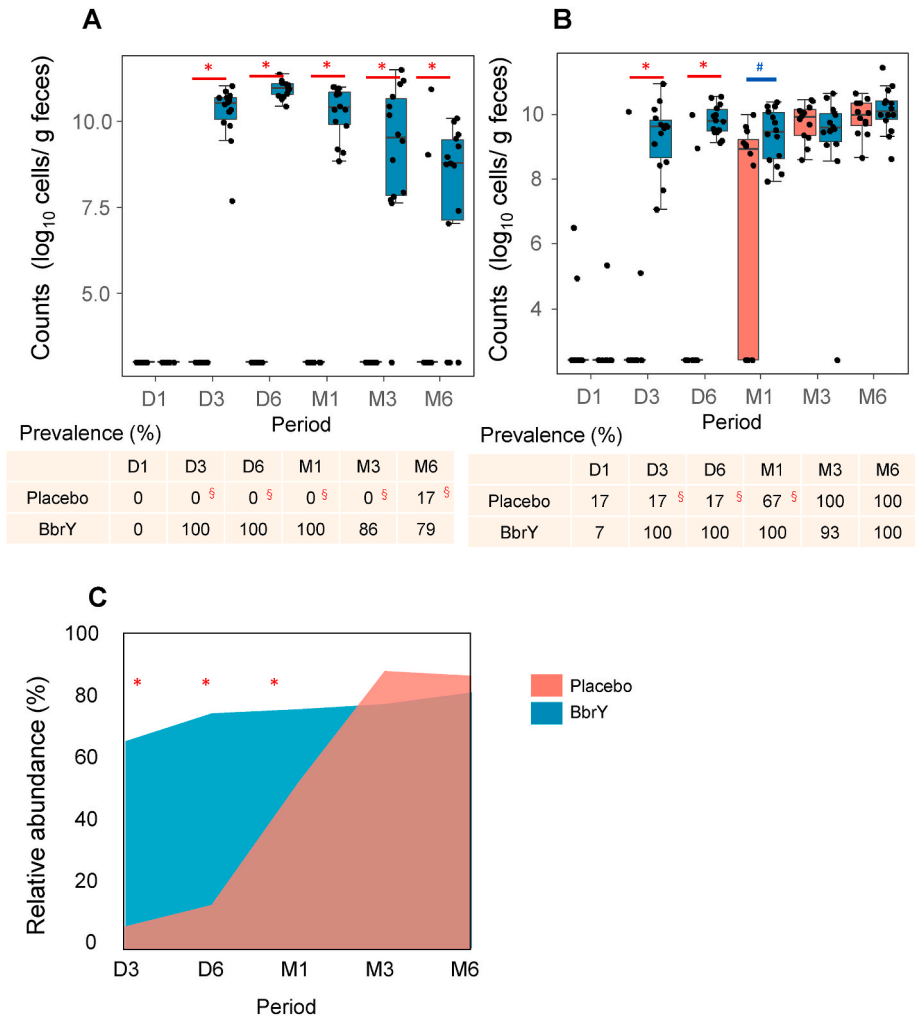


Fig. 2. Effects of BbrY administration on the counts and detection rates of BbrY (A) and *Bifidobacterium* (B) and the relative abundance of *Bifidobacteriaceae* (C). P-values were calculated using the Mann–Whitney U test (*p < 0.05; #p < 0.1) and Fisher's exact test (§p < 0.05).

6 days and higher counts of *Enterobacteriaceae* and *Veillonellaceae* at 3 months of age. The BbrY group also showed a trend toward lower counts of *Lactobacillaceae* in the first month and *Micrococcaceae* from day three to six months.

Supplementary Fig. 3 displays the changes in the α -diversity indices (Observed ASVs, Pielou evenness, and Shannon index), which showed significant differences or trends. Until the first month of life, the BbrY group had significantly lower or tended to have lower Observed ASVs, Shannon index, and Pielou evenness indices than the placebo group. Furthermore, no significant differences were observed in the counts of opportunistic pathogens or facultative anaerobes (e.g., *Clostridium perfringens*, *C. difficile*, *Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Pseudomonas*) at any time point (Supplementary Table 2). Fig. 3 shows the changes in organic acid concentration and

stool pH. The BbrY group had significantly higher concentrations of total organic acids, acetic acid, and lactic acid from day three to the first month of life than did the placebo group. Succinic acid concentrations also showed a significant increase or trend during the first and third months. Additionally, the BbrY group exhibited significantly lower stool pH than the placebo group from day three to the first month of life.

3.4. Effect of BbrY consumption on infant growth during the first year of life

Fig. 4 shows the changes in infant growth parameters (head circumference, height, weight, and Kaup index) during the first 12 months of life. The BbrY group showed a significantly higher mean weight at 9 months of age than the placebo group, as estimated using the

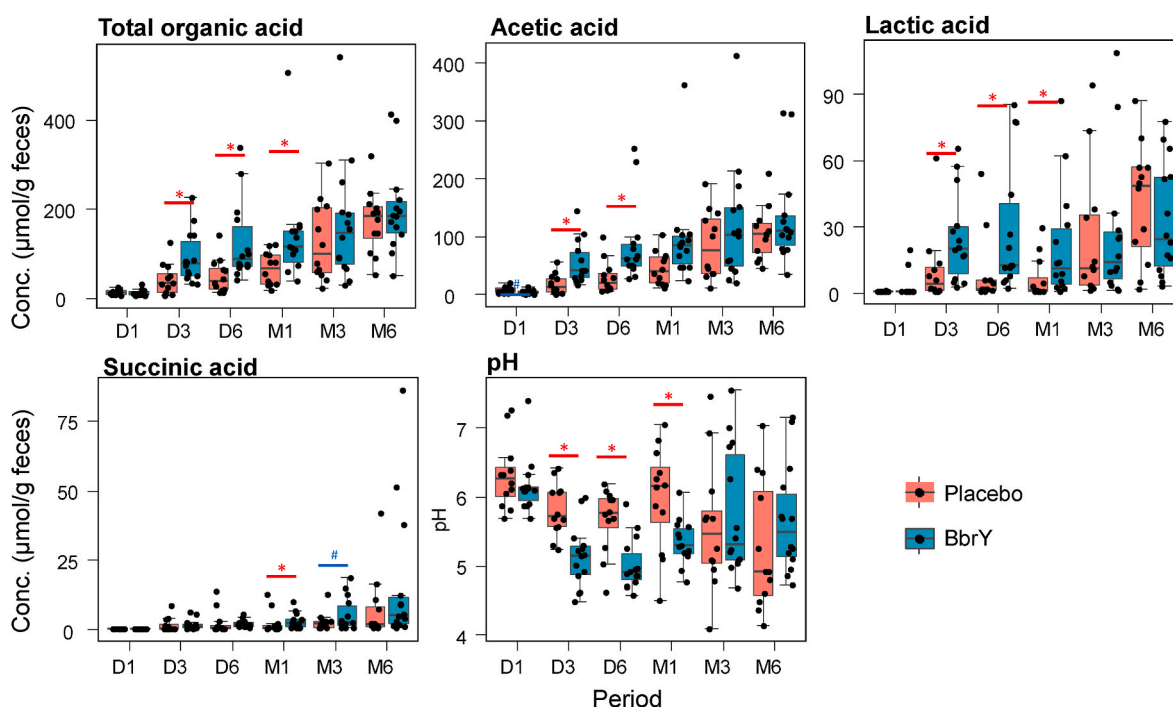


Fig. 3. Differences in fecal organic acid concentration and fecal pH changes between the BbrY and placebo groups during infancy. P-values were calculated using the Mann–Whitney U test ($\#p < 0.1$; $*p < 0.05$). Values below the detection limits were replaced with half of the limits. Detection limits for succinic acid, lactic acid, and acetic acid were set at 0.1, 0.1, and 0.1 $\mu\text{mol/g}$ feces, respectively.

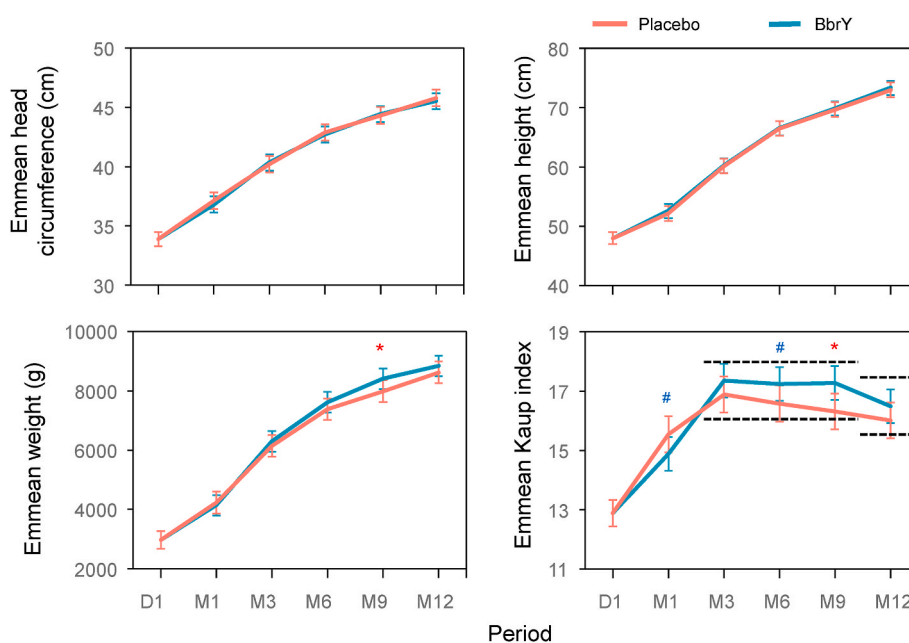


Fig. 4. Comparison of infant growth during the first year of life in the BbrY and placebo groups. Estimated baseline-adjusted means (emmean) and p-values were calculated using the mixed-effects model ($\#p < 0.1$; $*p < 0.05$). The error bars depict 95% confidence intervals, and the black dashed band represents the normal range of the Kaup index.

mixed-effects model. Additionally, the Kaup index in the BbrY group was significantly higher at 6 months and tended to be higher at 9 months. After 6 months of age, the lower limit of the 95% confidence interval for the Kaup index in the placebo group fell below the normal range, whereas it remained within the normal range in the BbrY group. No significant differences were observed in the other growth parameters between the two groups. [Supplementary Table 4](#) shows the frequency of illness and medication use in the first year of life. The number of illnesses

or medications or the proportion of participants who experienced no illnesses or medication use showed no significant differences between the two groups compared to those who experienced at least one instance of illness or medication use.

4. Discussion

Previous studies have indicated that infants born by cesarean section

exhibit delayed bifidobacterial colonization compared to vaginally delivered infants [3,21]. Our findings are consistent with these observations and demonstrate that BbrY supplementation promotes the early establishment of a *Bifidobacterium*-dominant microbiome during the first month of life in infants delivered by planned cesarean section. Although BbrY was still detected in 79% of infants at 6 months of age, this reflects partial persistence rather than permanent colonization following cessation of supplementation. In this study, we confirmed that in the first month of life, bifidobacteria were detected in 67% of infants in the placebo group at levels below 10^9 cells/g. These results suggest that bifidobacterial colonization, a key aspect in establishing the intestinal microbiota in neonates born vaginally, is also delayed in newborns delivered via planned cesarean section. In contrast, in the BbrY group, BbrY maintained a high detection rate and bacterial counts from day three to six months after birth (Fig. 2A). Additionally, the detection rate of *Bifidobacterium* and the relative abundance of the *Bifidobacteriaceae* family remained extremely high during the first month of life. Therefore, BbrY supplementation can help promptly establish a *Bifidobacterium*-dominated microbiota and address the delayed development of the gut microbiota in these infants. BbrY has been applied to several diseases in infants [22–25], and in some cases, early colonization by bifidobacteria in low-birth-weight infants has been reported [11,12]. However, no intervention trials have been conducted on full-term infants delivered via planned cesarean sections. To our knowledge, this study is the first to evaluate the effects of BbrY supplementation in this population, demonstrating its ability to promote the early establishment of a *Bifidobacterium*-dominant microbiome and facilitate the "normalization" of gut microbiota development in these infants.

More than 90% of the infants in this study were breastfed until the first month of life. BbrY possesses a solute-binding protein that selectively transports 4'-galactosyl lactose (4'-GL), a constituent of galactooligosaccharides found in human breast milk, into cells, thereby enabling the strain to grow vigorously by hydrolyzing and utilizing 4'-GL [26]. The most probable explanation for the predominance of *Bifidobacterium* in the neonatal gut microbiota of the BbrY group was the preferential utilization of undigested 4'-GL from breast milk by BbrY upon reaching the large intestine. Hence, in healthy cesarean-born infants, supplementation with BbrY may effectively promote the development of a *Bifidobacterium*-dominant microbiome during the early postnatal period without requiring commercial prebiotic supplementation.

Previous reports on the use of various bifidobacterial strains or oligosaccharides and *B. breve* administered immediately after birth showed less than 50% relative abundance of bifidobacteria at least one month after birth, with no reduction in α -diversity [27,28]. In contrast, in this study, several α -diversity indices in the BbrY group during the first month of life were significantly lower than those in the placebo group. The lower α -diversity in the BbrY group by the first month of life likely reflects the extreme predominance of *Bifidobacteriaceae*, particularly BbrY, in the intestines of infants. This finding suggests that BbrY supplementation may reduce microbial diversity in the short term as the microbiome becomes dominated by the administered strain.

We evaluated alterations in bifidobacterial composition using 16S rRNA gene sequencing. BbrY was the most frequently and abundantly detected strain in the BbrY group up to one month of age (Supplementary Fig. 4). However, after one month of life, BbrY was no longer predominant, and the bifidobacterial species distribution resembled that of the placebo group. This finding that BbrY is washed out after supplementation ends, which is an important safety feature, as it does not interfere with the natural growth of environmentally derived bifidobacteria.

In this study, the BbrY group had a total bacterial count $>10^{10}$ cells/g at 3–6 days of age, whereas the placebo group had a total bacterial count $<10^{10}$ cells/g. The total bacterial count in the gut of healthy infants born vaginally typically exceeds 10^{10} cells/g during the first week after birth [29]. Together, these findings suggest that the administration of BbrY

immediately after birth may help establish gut microbiota in cesarean-born infants at a rate comparable to that observed in vaginally delivered infants. Furthermore, the BbrY group showed an early increase in the concentration of total organic acids and a decrease in fecal pH. This was attributed to the increased concentrations of intestinal acetic and lactic acids during the early postnatal period. These metabolic changes were primarily attributable to bifidobacterial predominance, including BbrY, during the first month of life. Acetic and lactic acids are metabolites produced by bifidobacteria, and the presence of BbrY at a count of 10^{10} cells/g of feces and *Bifidobacteriaceae* comprising 70% of the microbiota likely contributed to these changes. It is conceivable that acetic and lactic acids produced by BbrY play a significant role in increasing the total organic acid concentration and reducing intestinal pH during the early stages of postnatal gut development. Correlation analyses further supported this interpretation: on day 3, BbrY abundance was strongly correlated with fecal pH ($\rho = -0.767$, $p = 0.001$) and organic acid concentrations (Supplementary Fig. 5). However, by day 6, these associations weakened to moderate trends (e.g., acetic $\rho = -0.525$, $p = 0.0537$; Supplementary Fig. 6), and were no longer significant at 1 month (Supplementary Fig. 7). This temporal pattern suggests that early acidification is closely associated with initial BbrY colonization but is subsequently maintained by a broader bifidobacterial community as strain dominance declines, consistent with previous reports describing the role of bifidobacteria and their metabolites in shaping intestinal pH during early life [6,7].

Previous studies have suggested that BbrY ingestion increases feeding volume and body weight in very low and extremely low birth weight infants [11,12]. Despite a trend toward lower birth weight in the BbrY group than that in the placebo group in this study, infants in the BbrY group had a significantly higher weight at 9 months. Moreover, after 3 months of age, the 95% confidence interval of the Kaup index for the placebo group deviated from the normal range, whereas that for the BbrY group remained within the normal range (Fig. 4). However, several studies on full-term cesarean-born infants ingesting bifidobacterial strains did not observe any significant effects of the administered strains [30,31]. In summary, BbrY is a unique strain that effectively promotes and regulates infant weight gain, aligning with the maturation of the gut microbiome.

5. Limitations

Our study has some limitations. First, this was a single-center study with a small sample size, which may have limited the ability to detect the full effect of BbrY on infant gut microbiota development and growth. Second, this study did not collect detailed data on complementary food composition; future studies should include comprehensive dietary records. Third, the small amount of DNA extracted from neonatal stool samples at birth precluded the amplification of 16S rRNA genes and our ability to characterize the initial microbial community. Alternative amplification methods are required to analyze the microbiota at birth more comprehensively. Fourth, the COVID-19 pandemic disrupted the timeline of this study by altering the hygienic environment surrounding childbirth and infant care. These changes may have influenced the development of gut microbiota and introduced confounding factors affecting microbial colonization patterns, which should be considered when interpreting our findings. Fifth, although the BbrY group demonstrated significantly higher weight at 9 months than the placebo group, this result should be interpreted cautiously. The study did not fully account for all potential confounding factors, such as feeding practices (e.g., formula feeding versus breastfeeding), socioeconomic status, and other environmental influences that could influence growth. Although BbrY supplementation may have contributed to improved growth, further studies are necessary to comprehensively understand its role. Finally, the long-term implications of early microbiome changes and growth differences remain unclear. This study did not evaluate associations between early microbiome changes and outcomes such as

gastrointestinal disorders or allergic diseases. Further longitudinal studies are required to determine how these early changes translate into long-term health and disease outcomes. Immune function was not assessed in this study; therefore, future research should include immunological endpoints to determine whether early BbrY supplementation confers immune benefits. Despite these limitations, our findings indicate that BbrY possesses a significant capacity to enhance the development of a *Bifidobacterium*-dominant microbiome. Future investigations should prioritize larger multicenter studies to validate the outcomes of this study.

6. Conclusion

Our study provides evidence that administering BbrY immediately after birth leads to the early establishment of a *Bifidobacterium*-dominant microbiome in cesarean-born infants with an immature microbiota at birth, similar to that observed in vaginally delivered infants. The rapid formation of an infantile-type microbiome contributes to the rapid production of acetic acid and acidification of the gut. Moreover, BbrY has the potential to support normal growth patterns and maintain Kaup index within the normal range of infants born via cesarean section. However, further research is required to better understand its role in supporting infant growth. Overall, these findings suggest that the administration of BbrY during the early stages of life may result in the establishment of a *Bifidobacterium*-dominant microbiome, which may contribute to promoting the future health of cesarean-born infants. Nevertheless, further research is needed to determine whether universal administration or targeted use in high-risk infants is most appropriate.

Data statement

Researchers interested in obtaining raw data on gut microbiota may submit a request to the corresponding author's institution.

Authors' contributions

Hirokazu Tsuji: Conceptualization, Methodology, Data analysis, Visualization, Writing- Original draft preparation. **Takashi Asahara:** Methodology, Data analysis, Writing- Reviewing and Editing. **Takafumi Sakai:** Investigation, Writing- Reviewing and Editing. **Yukiko Kado:** Data analysis, Validation. **Kaoru Moriyama-Ohara:** Data analysis, Validation. **Akira Takahashi:** Data analysis, Validation. **Rie Date:** Data analysis, Validation. **Satomi Atohe:** Data analysis, Validation. **Takako Ishizuka:** Data analysis, Validation. **Takuya Takahashi:** Investigation, Writing- Reviewing and Editing. **Saori Ozaki:** Sample and clinical data collection. **Yuko Shiono:** Sample and clinical data collection. **Yukihito Fukada:** Sample and clinical data collection. **Kazunari Kawashima:** Sample and clinical data collection. **Kazuo Yamashiro:** Writing- Reviewing and Editing. **Satoshi Matsumoto:** Conceptualization, Investigation, Writing- Reviewing and Editing. **Yuichiro Yamashiro:** Principal investigator, Supervision, Writing- Reviewing and Editing.

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Declaration of competing interest

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authors declare that there are no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pedneo.2026.02.001>.

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